Alteration of Chain Length Selectivity of a *Rhizopus delemar* Lipase Through Site-Directed Mutagenesis[1]

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The coding sequences of the *Rhizopus delemar* lipase and prolipase were altered by oligonucleotide-directed mutagenesis to introduce amino acid substitutions. The resulting mutant enzymes, synthesized by the bacterial host *Escherichia coli* BL21 (DE3), were tested for their ability to hydrolyze the triglycerides triolein (TO), tricaprylin (TC) and tributyrin (TB). Mutagenesis and lipase gene expression were carried out using plasmid vectors derived from previously described recombinant plasmids [Joerger and Haas (1993) *Lipids 28*, 81–88] by introduction of the origin of replication of bacteriophage f1. Substitution of threonine 83 (thr83), a residue thought to be involved in oxyanion binding, by alanine essentially eliminated lipolytic activity toward all substrates examined (TB, TO and TC). Replacement of thr83 with serine caused from two- to sevenfold reductions in the activity toward these substrates. Introduction of tryptophan (trp) at position 89, where such a residue is found in closely related fungal lipases, reduced the specific activity toward the three triglyceride substrates. For the mutagenesis of residues in the predicted acyl chain binding groove, mutagenic primers were designed to cause the replacement of a specific codon within the prolipase gene with codons for all other amino acids. Phenylalanine 95 (phe95), phe112, valine 206 (val206) and val209, were targeted. A phenotypic screen was successfully employed to identify cells producing prolipase with altered preference for olive oil, TC or TB. In assays involving equimolar mixtures of the three triglycerides, a prolipase with a phe95 → aspartate mutation showed an almost twofold increase in the relative activity toward TC. Substitution of trp for phe112 caused an almost threefold decrease in the relative preference for TC, but elevated relative TB hydrolysis. Replacement of val209 with trp resulted in an enzyme with a two- and fourfold enhanced preference for TC and TB, respectively.
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The fungus *Rhizopus delemar* [presently designated *R. oryzae* (1)] produces extracellular lipases that have been used in research for many years. One of these lipases has been purified and characterized (2), and a complementary DNA (cDNA) encoding this lipase has been cloned and its nucleotide sequence has been determined (3). This lipase, like its closely related counterpart produced by the fungus *Rhizomucor miehei* (4), exhibits a strong preference for the hydrolysis of ester bonds at the *sn*-1 and *sn*-3 positions of a triacylglycerol. As opposed to this pronounced positional selectivity, there is little selectivity for hydrolysis of substrates with different fatty acid moieties. In assays with triglyceride mixtures, tributyrin (TB) was the least preferred substrate. Triacylglycerols with longer chain fatty acids, both saturated and unsaturated, were hydrolyzed at only marginally different rates (5). In contrast, some lipases from *Candida cylindracea* exhibit little positional specificity (6), a lipase from *Geotrichum candidum* has a strong preference for the hydrolysis of *cis*-9 unsaturated fatty acids (6,7) and a lipase from *Penicillium camembertii* (Pc) hydrolyzes only mono- and diglycerides (8). The molecular bases for these selectivities are either still completely unknown or are just beginning to be revealed. Three-dimensional structures determined by X-ray crystallography are now available for several lipases (9–12), and continued refinement and analyses will provide new insights into the phenomenon of selectivity.

Rational mutagenesis provides another route for investigating the structure–function relationship of enzymes (13). Using this technique, it should be possible to gather useful information regarding the role of sequence and structure in determining the activity, selectivity and stability of these enzymes. In order to facilitate studies on the *R. delemar* (Rd) lipase, the cloned cDNA encoding this lipase was used to construct expression plasmids for the high-level production of the proenzyme and mature form of the lipase in the bacterium *Escherichia coli* (14).

We have now modified the expression plasmids to create plasmids that permit site-directed mutagenesis and expression to be carried out with the same vector. This system was used to create mutations in the lipase and prolipase genes and to express these altered genes. The gene products were then tested for their ability to hydrolyze TB, tricaprylin (TC) and triolein (TO).

MATERIALS AND METHODS

DNA manipulations. Plasmid DNA was isolated by an alkaline lysis procedure (15) followed by phenol/chloroform extraction. Single-stranded DNA was isolated from cul-

[1]Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Abbreviations: aa, Amino acid; ala, alanine; asp, aspartate; cDNA, complementary deoxyribonucleic acid; gly, glycine; Hl, *Humicola lanuginosa*; IPTG, isopropyl-β-D-thiogalactoside; leu, leucine; Pc, *Penicillium camembertii*; phe, phenylalanine; Rm, *Rhizomucor miehei*; Rd, *Rhizopus delemar*; ser, serine; TB, tributyrin; TC, tricaprylin; TE buffer, Tris/ethylenediaminetetraacetic acid buffer; thr, threonine; TO, triolein; Tris, tris(hydroxymethyl)aminomethane; trp, tryptophan; val, valine.

ture fluids by precipitation with 1/4 vol of 20% polyethylene glycol in 3.75 M ammonium acetate (NH_4OAc). The precipitate was resuspended in Tris/ethylenediaminetetraacetic acid (TE) buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, pH 8) and extracted repeatedly with phenol/chloroform. DNA was stored in TE buffer. Restriction enzyme digestions and ligations were performed as recommended by the suppliers. Plasmid DNA was introduced into *E. coli* cells either by transformation of $CaCl_2$-treated cells (16) or by electroporation using a Gene Pulser™ (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's directions. Sequencing of DNA was carried out by the method of Sanger *et al.* (17) using the Sequenase™ Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH).

Vector construction. The origin of replication of bacteriophage f1 was introduced into the lipase expression plasmid pET11-d-431 (14) in a procedure similar to that described by Richardson and Richardson (18). The f1 origin of replication was isolated from plasmid pTZ18R (purchased from Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as a 1060-bp *Sca*I-*Hind*III fragment. Plasmid pET11-d-431 was cleaved with restriction enzyme *Sca*I and the linearized plasmid incubated with *Hind*III under conditions favoring partial digestion. The resulting fragments were separated by agarose gel electrophoresis, and the band corresponding to a *Sca*I-*Hind*III fragment approximately 6 kbp in size was excised from the gel, extracted and purified. This fragment was ligated to the f1 ori-containing *Sca*I-*Hind*III fragment from pTZ18R to yield a plasmid, pET11-d-f1-431, that retained the features required for lipase gene expression and also allowed production of single-stranded circular DNA for use in site-directed mutagenesis.

Under inducing conditions, cells harboring pET11-d-f1-431 produce inactive lipase that has to be refolded into active enzyme. Direct production of active mature Rd lipase in *E. coli* is not possible due to the toxic nature of this enzyme. However, a plasmid allowing expression of active prolipase in *E. coli* BL21 (DE3) (19) was constructed. To achieve this, the *Eco*RI-*Xba*I fragment from pET11-d-f1-431, which contains the gene for mature lipase, was replaced with an *Eco*RI-*Xba*I fragment encoding prolipase. This fragment was isolated from plasmid pTM-N-1231, which consists of vector pTM-N (20) and the Rd prolipase gene fused to DNA encoding the OmpA signal peptide. The new plasmid, pET11-d-f1-1231s, was used for isolation of single-stranded DNA and for production of active prolipase.

Plasmids for the production of mature lipases carrying the amino acid substitutions found in previously characterized prolipases were constructed by replacement of the 600-bp *Kpn*I-*Eco*RI fragment in pET11-d-f1-431 with the corresponding fragments from the pET11-d-f1-1231s mutant plasmids. These pET11-d-f1-431 derivatives were used for the production of mature mutant lipases in inactive forms as inclusion bodies. Active enzyme was obtained after refolding as described previously (14).

Production of single-stranded DNA from pET11-d-f1-431 and pET11-d-f1-1231s. Single-stranded DNA was obtained by infecting plasmid-containing *E. coli* JM101 cells with helper phage M13KO7 as described by Viera and Messing (21). Packaged single-stranded DNA was precipitated with PEG/NH_4OAc and purified by repeated phenol/chloroform extractions. For the production of single-stranded DNA for use in mutagenesis experiments according to the methods of Kunkel (22), plasmids were first introduced into *E. coli* strain CJ236. Uracil-containing single-stranded DNA was prepared from the culture supernatant of M13KO7-infected cells as indicated above.

Oligonucleotide-directed mutagenesis. Site-directed mutagenesis was carried out by the method of Kunkel (22) using the Muta-Gene M13 *in vitro* Mutagenesis Kit (Bio-Rad Laboratories). Mutagenic primers were obtained from the Department of Chemistry, University of Pennsylvania (Philadelphia, PA). The nucleotide sequences of the primers used are listed in Table 1. Primer-mediated nucleotide sequence changes affecting restriction enzyme recognition sites were so chosen as not to alter the amino acid sequence of the lipases.

TABLE 1

Mutagenic Primers

Primer nucleotide sequence[a]	Amino acid change introduced[b]	Other changes introduced[c]
5′TCCGTGGTGCTAACTCCTTC3′	thr83- > ala	removal of *Kpn*I site at pos. 658
5′TCCGTGGTTCCAACTCCTTC3′	thr83- > ser	removal of *Kpn*I site at pos. 658
5′TCAGAAGTTGGATCACTGACATCGTCTTC3′	ala89- > trp	removal of *Eco*RV site at pos. 688
5′CACTGATATTGTCNNXAACTTTTCT3′	phe95- > aa	removal of *Eco*RV site at pos. 688
5′AGTTCATGCCGGCNNXCTTTCCTCT3′	phe112- aa	introduction of *Nae*I site at pos. 742
5′GAGAGATATTNNXCCTCACN NXCCTCCTCAA3′	val206- aa, val209- aa	removal of *Eco*RV site at pos. 1024

[a]During primer synthesis, an equimolar amount of A, C, G and T was used at the positions indicated by the letter N. Only equimolar amounts of C and G were used at the positions indicated by letter X.

[b]Amino acids are numbered as in Reference 3; aa indicates that any one of the twenty possible amino acids could be present after mutagenesis. Abbreviations: thr, threonine; ala, alanine; ser, serine; trp, tryptophan; phe, phenylalanine.

[c]The pos. numbers are as in Reference 3 and denote the position of the first nucleotide of the restriction enzyme recognition site.

Mutant screening. Mutated versions of pET11-d-f1-431 were identified initially by acquisition or loss of a restriction enzyme site (see Table 1). DNA sequencing was then used to confirm the presence of the desired base changes. A phenotypic screening for cells carrying mutated versions of pET11-d-f1-1231s consisted of replica-plating cells onto solid LB-agar medium (rhodamine B medium) containing dispersed triglyceride (25 mL/L), ampicillin (100 μg/mL), isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mM) and rhodamine B (0.0005%) (3). Fatty acids produced by lipase activity cause the rhodamine B to acquire a bright pink color and to fluoresce under ultraviolet light. Cells acquire a similar coloration and become surrounded by halos. Lipase negative cells remained cream-colored. Plasmid DNA was isolated from cells producing mutant lipases of interest for this study, and the nucleotide sequence of the region expected to carry a mutation was determined.

Induction of lipase gene expression. Escherichia coli BL21 (DE3) harboring recombinant plasmids were grown on solid LB medium (23) containing ampicillin (100 μg/mL). Cells containing pET11-d-f1-431 or its mutagenized derivatives were cultured in liquid medium and induced for lipase expression by the addition of IPTG to a final concentration of 1 mM as previously described (14). Cells containing pET11-d-f1-1231s or its mutant forms were induced in liquid culture (2 to 10 mL of LB medium containing ampicillin) by the addition of IPTG to a final concentration of 0.2 mM when the optical density (at 600 nm) of the culture reached 0.7 to 1.0. Cells were harvested 3 to 4 h after the addition of inducer.

Preparation of mature and prolipase samples. Wild-type and mutant versions of mature lipase were refolded and purified, and their purity and specific activities were determined as previously described (14). Briefly, the lipase inclusion bodies were solubilized in 8 M urea, and refolding was initiated by dilution. The refolded lipase preparation was purified to homogeneity by affinity and ion-exchange chromatography. Purity was assessed visually by examination of acrylamide electrophoresis gels stained with Coomassie Blue.

Cells induced for prolipase production were resuspended in 20 mM sodium phosphate buffer, pH 6.5, and sonicated. The lysate was used directly in lipase activity assays.

Determination of lipase activity. Activity determinations were carried out titrametrically as described previously (14) using a VIT 90 Video titrator (Radiometer, Copenhagen, Denmark) and 0.1 N NaOH as titrant. The assays were conducted at 25°C at pH of 7.5. The reaction mixture consisted of 200 mM olive oil, TC or TB (Sigma, St. Louis, MO) in 4.2% aqueous gum arabic solution and 14 mM $CaCl_2$. Lipase activity was calculated from the maximum rate of titrant addition using a lipase titrametric assay program (Cichowicz, D.J., *et al.,* unpublished). A unit (U) of activity released one μmole of fatty acid per minute.

Substrate selectivity was determined in an assay containing 500 μL of 50 mM sodium phosphate, pH 7.5, and 200 μmoles each of TO, TC and TB. The reactions were started by the addition of purified mature enzyme or prolipase-containing lysates. Samples to which only buffer or heat-inactivated cell sonicate had been added served as controls. The tubes were incubated at 30°C on an orbital shaker that was rotated at 300 rpm. After two hours, 20 μmoles of palmitic acid in 50 μL pyridine was added to the tubes to serve as internal standard, the content of the tubes was thoroughly emulsified, and 10 μL was quickly withdrawn to another tube. Then, 100 μL of dry pyridine was added, followed by 400 μL of *bis*(trimethylsilyl)trifluoroacetamide (Sigma). The tubes were incubated for 15 min in a boiling waterbath (24), cooled to room temperature, and hexane was added to obtain a solution appropriately diluted for gas chromatography. The samples were injected on-column into a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph containing a 15-m nonpolar high-temperature column (DB1-HT) with an inner diameter of 0.32 mm and a film thickness of 0.1 μm (J&W Scientific, Folsom, CA). The helium flow rate was 5.5 mL per min. Detection was by flame ionization. The oven temperature was maintained at 40°C for 0.5 min, then increased to 100°C at a rate of 20°C per min, and then increased to 120°C at a rate of 2°C per min. The temperature was finally raised to 350°C at a rate of 20°C per min. This triphasic temperature profile was necessary to separate TB from monocaprylin. Data collection and processing was done on a Hewlett-Packard 3396 SeriesII Integrator.

RESULTS AND DISCUSSION

Mutagenesis experiments. The system for the expression of the Rd prolipase and mature lipase genes in *E. coli* described previously (14) allowed the synthesis and subsequent purification of relatively large amounts of these lipases. The modification of the expression vectors to include the bacteriophage f1 origin of replication was undertaken to produce vectors that can be used to bring about mutagenesis and expression without cloning in two different vectors. This approach had been proven successful previously in studies of the gene for the transcription termination factor, rho (18).

In initial experiments, threonine (thr) 83 was replaced with either alanine (ala) or serine (ser). Thr83 occupies the same position in the Rd lipase as ser82 in the *Rhizomucor miehei* (Rm) lipase (4). This residue has been implicated in oxyanion binding during hydrolysis of triglycerides by the Rm lipase (9,10). Cells harboring mutagenized plasmids were induced to produce inactive lipase in the form of inclusion bodies. The mutant lipases were then refolded, purified and characterized (14). Replacement of thr83 with ala caused a decrease in the specific activity by almost three orders of magnitude (Table 2). A similar loss of activity was observed when a corresponding ser residue was replaced by glycine (gly) in the mono- and diglyceride lipase from Pc (25). These reductions in activity are not unexpected because the loss of anion stabilization would be expected to profoundly reduce the activity of the enzyme. Neither gly nor ala can supply a hydroxyl group to attain this stabilization. On the other hand, replacement of thr83 with ser resulted in a lipase whose specific activity on various substrates was between one-fifth and one-half that of the wild type (Table 2). Presumably, the ser hydroxyl can partially substitute for the thr hydroxyl, but

TABLE 2

Titrametric Analysis of Lipolytic Activity of Refolded and Purified Recombinant Lipases

Source[a]	Mutation[b]	Specific activity[c]		
		Olive oil	Tricaprylin	Tributyrin
pET11-d-f1-431	wild type	3149	8539	2944
pET11-d-f1-431-T83A	thr83- > ala	4	7	1
pET11-d-f1-431-T83S	thr83- > sér	1664	3350	442
pET11-d-f1-431-A89W	ala89- > trp	2106	6628	1702

[a]Expression plasmid present in *Escherichia coli* BL21 (DE3).

[b]Amino acids numbered as in Reference 3. See Table 1 for abbreviations.

[c]Micromoles fatty acid released per min per mg of protein. The values are averages of two experimental determinations.

the difference in the positioning of the side chains of the two amino acids is significant enough to reduce the specific activity of the ser variant.

Residues 86 to 92 in the Rd lipase are predicted to form a "lid" that would cover the active site of the lipase, as was shown by crystallographic studies of the related Rm lipase (9,10). It has been postulated, and demonstrated using inhibitors (9,10), that this lid is displaced when substrate analogues bind to the active site. This displacement exposes the active site and increases the potential lipase-substrate interface by enlarging the hydrophobic surface area of the enzyme. These features are thought to underlie the requirement, demonstrated by all lipases, that an interface be present before activity is manifested. Ala89 occupies a position in the Rd lipase where tryptophan (trp) is found in the lipases from *R. miehei, Humicola lanuginosa* (Hl) and Pc (25; Derewenda, Z.S., personal communication). It was postulated that this trp might play a role in the interfacial activation process (9). Recently, Clausen *et al.* (26) reported that in the Rm lipase, replacement of the trp did not abolish interfacial activation, but that substrate penetration was changed. By replacing ala89 in the Rd lipase with trp, we attempted to obtain more insight into the role of hydrophobic residues in the lids of these fungal lipases, especially as it relates to enzyme–substrate interaction. We found that replacement of ala89 with trp caused a 22 to 42% reduction in the specific activity relative to the corresponding activity of the purified wild-type lipase (Table 2). However, compared to the wild-type enzyme there was no profound change in the relative activity of this modified enzyme toward the various substrates tested (Tables 2, 3; Figs. 2, 3). Further studies, such as determination of the interfacial activation curve of the wild-type and mutant enzymes, and elucidation of the three-dimensional structures of these proteins will be required in order to precisely identify the roles of specific amino acids in the lid of the enzyme.

The plasmid system described above for the mutagenesis and expression of the thr and ala mutants has the advantage of supplying relatively large quantities of pure lipase, but the disadvantage of requiring isolation of the inactive lipase as inclusion bodies with subsequent refolding to produce active enzyme. Despite many attempts to find an expression system that would allow the production of active, mature lipase, no system could be developed that was able to overcome the toxic effect of this enzyme on the bacterial host (14). Thus, a plasmid that allows the production of active prolipase, which appears to be less toxic, was constructed for the experiments with mutagenic primers containing a randomized codon (Table 1). Our goal was to determine whether or not it was possible to alter the substrate specificity of the Rd lipase by replacement of specific amino acid residues. The residues chosen [phenylalanine (phe) 95, phe112, valine (val) 206, val 209], are well conserved in the Rd, Rm, Hl and Pc enzymes and are predicted to reside in a groove that might accommodate the acyl chain of a substrate glyceride bound in the active site (9,10) (Fig. 1).

TABLE 3

Hydrolysis of an Equimolar Mixture of Triolein, Tricaprylin and Tributyrin by Refolded, Purified Recombinant Mature Lipases

Source	µg of lipase in assay	% Hydrolyzed[a]		
		Triolein	Tricaprylin	Tributyrin
pET11-d-f1-431	2.5	80.1	83.9	42.4
pET11-d-f1-431-T83A	435.0	21.8	24.2	5.7
pET11-d-f1-431-T83S	2.7	58.0	56.4	10.2
pET11-d-f1-431-A89W	2.5	60.4	72.6	17.4

[a]Percent hydrolyzed = $(TG_C - TG_H)/(TG_C) \times 100$ where TG_C equals the amount of triglyceride present in enzyme-free reaction and TG_H is the amount of triglyceride remaining after incubation with the lipase.

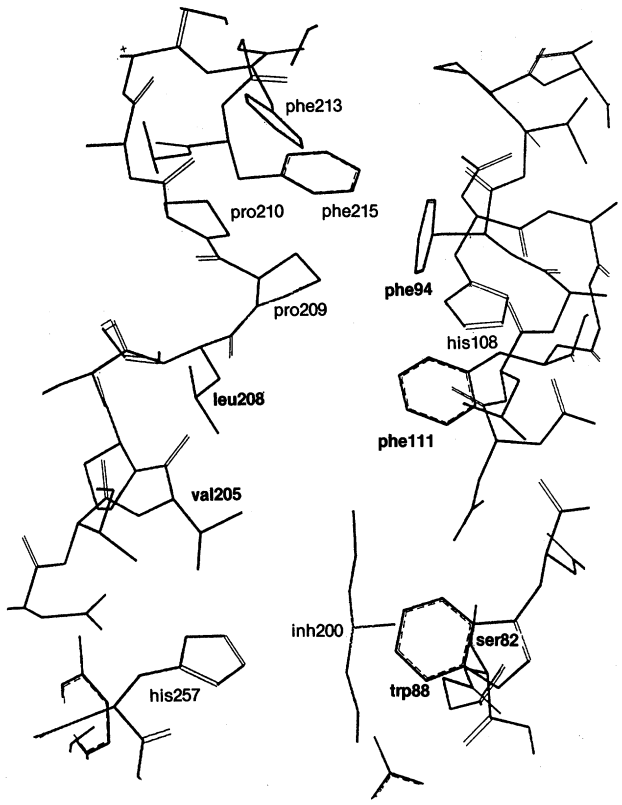

FIG. 1. Structure of a part of the *Rhizomucor miehei* (Rm) lipase with the active site inhibitor (INH200) *n*-hexylphosphonate ethylester (9). The oxyanion-binding ser82 as well as residues lining the fatty acid-binding groove are shown. The corresponding residues in the *Rhizopus delemar* lipase are thr83 in place of Rm (ser82), phe95 (phe94), his109 (his108), phe112 (phe111), val206 (val205), val209 (leu208), pro210 (pro209), pro211 (pro210), phe214 (phe213), phe216 (phe215), his257 (his257). Crystallographic coordinates kindly provided by Z. Derewenda. Abbreviations: ser, serine; thr, threonine; phe, phenylalanine; val, valine; pro, proline; his, histidine.

To scan large numbers of mutants, the phenotypic lipase assay of Kouker and Jaeger (27) was employed. In this procedure, colonies arising after mutagenesis were replicated onto rhodamine medium (see Materials and Methods section). Thus colonies could be identified that differed from the wild type in their capacity to hydrolize the components of olive oil or TC. This phenotype was ascertained to be stable by retesting on the rhodamine B media. The majority of colonies arising after mutagenesis had an appearance on rhodamine B media that was indistinguishable from that of wild-type colonies. This is due in part to mutagenesis efficiencies of less than 100%. However, the wild-type phenotype predominated even among colonies that contained mutated plasmids as indicated by the occurrence of a mutagenic primer-induced restriction enzyme susceptibility. This suggests that, despite the conserved character of the residues subjected to mutagenesis, a number of replacements are tolerated without a major change in the lipolytic phenotype. The nature of these neutral substitutions is not known because no sequence determinations were made. A second group of mutants exhibited a lipase-negative phenotype. Presumably, some of the substituting amino acids have side chains that prevent either proper folding of the enzyme or the conformational changes required for interfacial activation, substrate binding or catalysis.

Hydrolysis of olive oil, TO, TC and TB. The activities of mutant lipases were tested titrametrically against the individual substrates. For the pure wild-type and mutant mature enzymes, specific activities could be assigned for each substrate (Table 2) and a profile of the relative activities, with the activity against olive oil set at 100%, was established (Fig. 2). The reduction in the specific activity as a result of the replacement of thr83 by ser or ala has been noted previously. In addition to this reduction in activity, these mutant enzymes display an alteration in their substrate preferences, with the activity toward TB, relative to that toward TO, being reduced roughly threefold compared with the wild-type enzyme (Fig. 2). The titrametric assay using individual substrates allowed the relatively rapid screening of potential mutant lipases and provided information concerning the activities of the enzymes. However, to overcome differences in the emulsion properties of TB, TC and olive oil, experiments were also conducted that presented the enzymes with mixtures of three substrates. Activity was determined by measuring the disappearance of each substrate during incubation with the enzymes. TO was used in place of olive oil in these assays to simplify the gas-chromatographic analyses. As with the wild-type enzyme, purified mutant lipases had similar activities toward TC and TO (Table 3). However, compared to the wild-type enzyme, the thr83 and ala89 mutant lipases showed a reduction in their relative activities toward TB (Table 3, Fig. 3).

The lipolytic activities, toward individual triglyceride substrates, of lysates containing wild-type or mutant prolipases are listed in Table 4. Since these data were collected using impure enzyme preparations, the activities are listed in units per mL of cell lysate, and absolute activity values cannot be compared with one another. Nevertheless, the relative activities can be compared (Fig. 4A). As seen with the mature wild-type lipase (Table 2, Fig. 2),

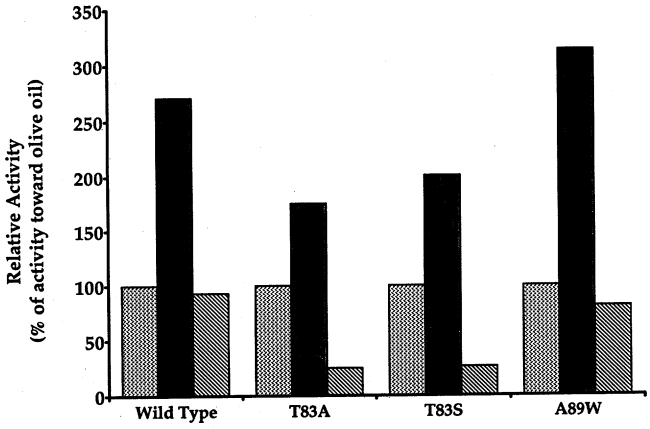

FIG. 2. Relative activities calculated from the results of the titrametric assays on olive oil (dotted bars), tricaprylin (dark bars), and tributyrin (hatched bars), listed in Table 2. The activity on olive oil was set at 100%.

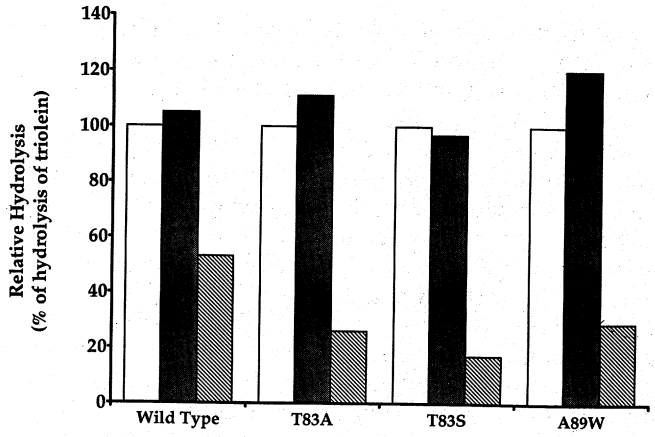

FIG. 3. Relative activities calculated from the results, listed in Table 3, of hydrolysis experiments with substrate mixtures composed of triolein (light bars), tricaprylin (dark bars) and tributyrin (hatched bars). The percent hydrolysis of triolein was set at 100%.

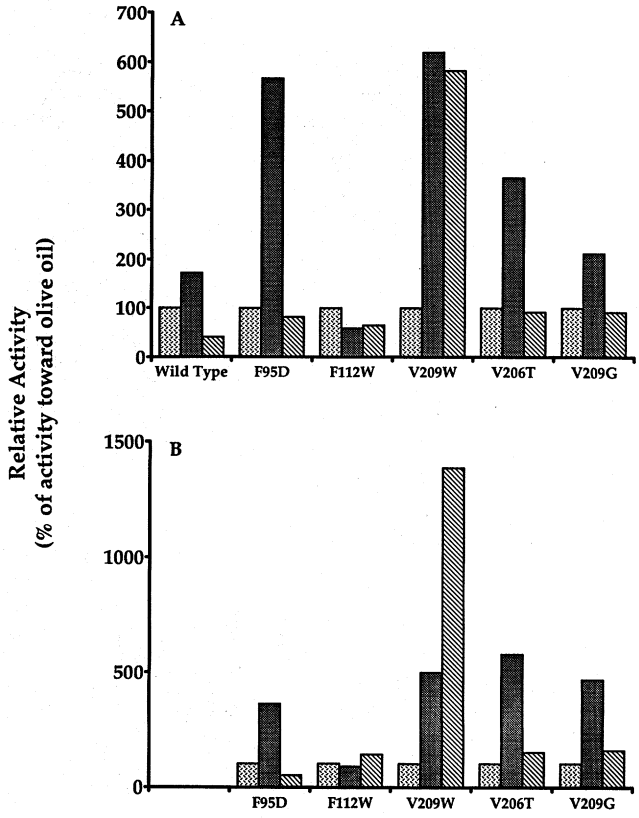

FIG. 4. The relative lipolytic activities of prolipase (A) and mature lipase (B) mutants against the single substrates olive oil (dotted bars), tricaprylin (dark bars) and tributyrin (hatched bars). Activity on olive oil was set at 100%. Data from Tables 4 and 6, respectively.

the wild-type prolipase is most active with TC as substrate and least active against TB. This was confirmed by incubation of the enzymes in the simultaneous presence of the three substrates (Table 5, Fig. 5A). However, there was only a small difference between the hydrolysis of TO and TC in this case as opposed to the experiments using single substrates (Table 4).

The prolipase expressed from pET11-d-f1-1231s-F95D exhibits more than a threefold higher activity toward TC than does the wild enzyme in the single substrate titration assay (Table 4, Fig. 4A), and an almost twofold higher activity in the mixed substrate assay (Table 5, Fig. 5A). Whether this is caused by a higher hydrolytic activity toward TC or a reduced activity toward TO cannot be determined with these impure preparations.

Replacement of phe112 by trp greatly effected the substrate preference of the prolipase. In contrast to the wild type, the lysate from a strain harboring pET11-d-f1-1231s-F112W exhibited its lowest activity toward TC (Tables 4, 5). Relative to the wild-type enzyme, this mutant enzyme hydrolyzed TB about 50% better, with the ratio of TO activity to TB activity being 1.6, compared to 2.4 for the wild type (Table 4, Fig. 4A). Against mixed substrates, TC was again least preferred, while TB was hydrolyzed to a large extent (Table 5, Fig. 5A). Thus the mutagenic replacement of a large side chain by an even bulkier one reduced the relative activity toward triglycerides with medium-chain fatty acids and increased activity toward those with short chains.

Mutant plasmid pET11-d-f1-1231s-V209W encodes a prolipase with a substitution of val209 by a trp residue. *Escherichia coli* cells bearing this plasmid produced a prolipase with highest activity on TB in the mixed substrate

TABLE 4

Lipolytic Activity in Lysates of Prolipase-Producing *Escherichia coli* BL21 (DE3)

Source	Mutation	Activity (U/mL)[a]		
		Olive oil	Tricaprylin	Tributyrin
pET11-d-f1-1231s	wild type	158	270	65
pET11-d-f1-1231s-F95D	phe95- > asp	35	198	29
pET11-d-f1-1231s-F112W	phe112- > trp	100	59	65
pET11-d-f1-1231s-V209W	val209- > trp	16	99	93
pET11-d-f1-1231s-V206T	val206- > thr	64	234	41
pET11-d-f1-1231s-V209G	val209- > gly	54	114	50

[a]Data presentation as in Table 2, but activities expressed as U per mL of lysate from induced cells; asp, aspartate; val, valine; gly, glycine; see Table 1 for other abbreviations.

TABLE 5

Hydrolysis of Equimolar Mixtures of Triolein, Tricaprylin and Tributyrin by Prolipase in Cell Lysates

Source	Mutation	% Hydrolysis[a]		
		Triolein	Tricaprylin	Tributyrin
pET11-d-f1-1231s	wild type	66.8	75.8	39.0
pET11-d-f1-1231s-F95D	phe95- > asp	36.9	67.7	34.1
pET11-d-f1-1231s-F112W	phe112- > trp	18.4	7.2	67.7
pET11-d-f1-1231s-V209W	val209- > trp	18.2	35.4	72.0
pET11-d-f1-1231s-V206T	val206- > thr	66.1	72.4	43.0
pET11-d-f1-1231s-V209G	val209- > gly	54.8	60.0	60.0

[a]Calculated by the method defined in Table 3. Abbreviations as in Tables 1 and 4.

assay (Table 5, Fig. 5A) and toward TC and TB in the single substrate assay (Table 4, Fig. 4A). Apparently, this enzyme has a relatively low activity on TO, but as before, it will only be possible to verify this through purification of the prolipase. As with the mutant prolipase (F112W) described above, the change in substrate preference of the V209W mutant prolipase toward the substrate with the shortest acyl chains, TB, was caused by the replacement of a hydrophobic amino acid, val, with a bulky residue, trp.

The replacement of val206 with thr, and the replacement of val209 with gly, caused an increase in the relative activity of the prolipases toward TC in the single substrate assay (Fig. 4A). However, in the mixed substrate assay, the V206T mutant prolipase hydrolyzed the three substrates in a manner similar to that of the wild-type enzyme and the V209G mutant prolipase hydrolyzed each of the three substrates almost equally (Fig. 5A).

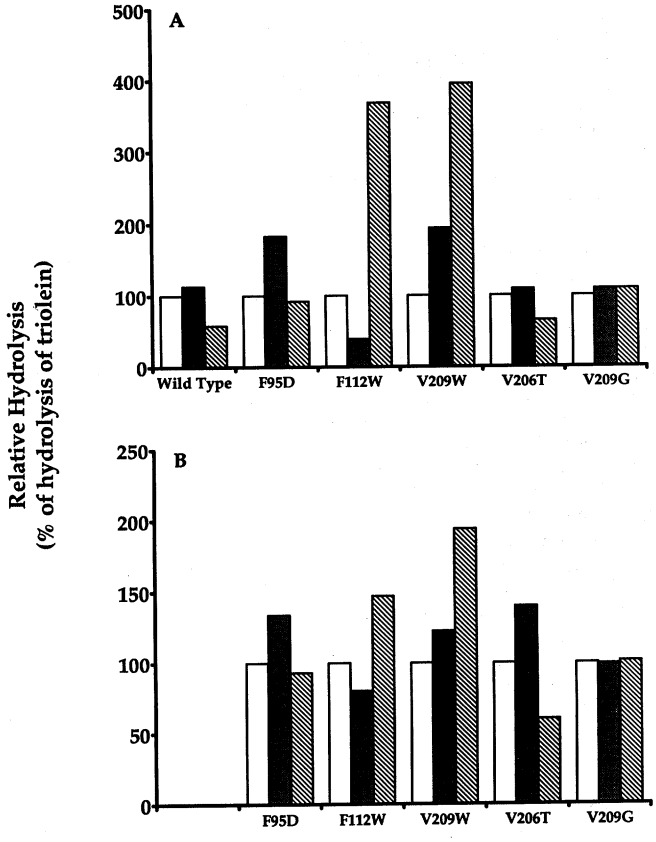

FIG. 5. The relative activities of wild-type and mutant lipases against mixtures of the substrates triolein (light bars), tricaprylin (dark bars) and tributyrin (hatched bars); (A) prolipase, data taken from Table 5; (B) mature lipase, data taken from Table 7. The activity toward triolein was set at 100%.

The production of mutant lipases as active proenzymes is an efficient way to rapidly screen for enzymes with altered properties. The presence of the propeptide and the need to use crude preparations, however, raises the question as to whether the properties observed are also those that would be observed with the corresponding mature enzymes. In order to address this question, the mutations carried by the prolipase genes were introduced into the gene for mature lipase by replacement of a *Kpn*I-*Eco*RI fragment from this gene with the corresponding fragments from the mutated prolipase genes. The resulting mutant lipase genes were expressed, and active mutant lipase enzymes were obtained after refolding of the inactive enzymes from inclusion bodies. Specific activities were obtained for the partially purified lipase preparations for the single substrates olive oil, TC and TB (Table 6). The relative activities (activity toward olive oil equals 100%) are shown in Figure 4B. Comparison of the data in Figure 4A and 4B indicates that the crude prolipase preparations and the partially purified refolded mature lipase samples behave similarly. Differences in the relative activities toward TC, mainly by the V209W mutant, however, could be

TABLE 6

Titrametric Analysis of Lipolytic Activity of Partially Purified, Refolded Recombinant Mature Lipases

Source[a]	Specific activity[b]		
	Olive oil	Tricaprylin	Tributyrin
pET11-d-f1-431-F95D	821	2978	404
pET11-d-f1-431-F112W	1279	1127	1800
pET11-d-f1-431-V209W	281	1398	3896
pET11-d-f1-431-V206T	187	1079	281
pET11-d-f1-431-V209G	187	466	296

[a]Plasmid present in *Escherichia coli* BL21 (DE3).

[b]Micromoles fatty acid released per min per mg of protein. The values are averages of two experimental determinations.

TABLE 7

Hydrolysis of Equimolar Mixtures of Triolein, Tricaprylin and Tributyrin by Partially Purified, Refolded Mutant Mature Lipases

Source	% Hydrolysis[a]		
	Triolein	Tricaprylin	Tributyrin
pET11-d-f1-431-F95D	59.3	79.5	55.3
pET11-d-f1-431-F112W	40.3	32.1	58.7
pET11-d-f1-431-V209W	34.3	42.5	66.4
pET11-d-f1-431-V206T	15.6	21.3	8.9
pET11-d-f1-431-V209G	47.1	46.7	47.6

[a]Values are average from two assays; 5 μg of protein was added to each reaction tube.

noted. The reasons for these differences remain to be elucidated.

The refolded mutant lipase samples were also tested in mixed substrate assays (Table 7). As seen in the single substrate assay, the relative hydrolysis data for the mutant prolipases and the mature mutant lipases closely resembled each other (Fig. 5).

Overall, the phenotypes observed so far indicate that it is possible to manipulate the substrate specificity of the Rd lipase through site-directed mutagenesis. Additional mutagenesis experiments, involving such targets as phe214 and phe216 (see Fig. 1), or deletions or insertions near residues that might interact with the acyl chain of triglycerides, should increase the spectrum of mutants. The combining of different mutations into one mutant lipase might yield enzymes with even higher substrate preferences. The determination of the three-dimensional structure of the Rd lipase (28), should allow the identification of additional mutagenesis targets and possibly provide explanations for the phenotypes caused by the mutations studied here.

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REFERENCES

1. Schipper, M.A.A. (1984) *Studies in Mycology 25,* 1–19.
2. Haas, M.J., Cichowicz, D.J., and Bailey, D.G. (1992) *Lipids 27,* 571–576.
3. Haas, M.J., Allen, J., and Berka, T.R. (1991) *Gene 109,* 107–113.
4. Boel, E., Huge-Jensen, B., Christensen, M., Thim, L., and Fiil, N.P. (1988) *Lipids,* 701–706.
5. Huge-Jensen, B., Rubano Galluzzo, D., and Jensen, R.G. (1988) *J. Am. Oil Chem. Soc. 65,* 905–910.
6. Jensen, R.G., Rubano Galluzzo, D., and Bush, V.J. (1990) *Biocatalysis 3,* 307–316.
7. Jensen, R.G. (1974) *Lipids 9,* 149–157.
8. Yamaguchi, S., and Mase, T. (1991) *Appl. Microbiol. Biotechnol. 34,* 720–725.
9. Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.B., Bjorkling, F., Huge-Jensen, B., Patkar, S.A., and Thim, L. (1991) *Nature 351,* 491–494.
10. Derewenda, Z.S., Derewenda, U., and Dodson, G.G. (1992) *J. Mol. Biol. 227,* 818–839.
11. Schrag, J.D., Li, Y., Wu, S., and Cygler, M. (1991) *Nature 351,* 761–764.
12. Grochulski, P., Li, Y., Schrag, J.D., Bouthillier, F., Smith, P., Harrison, D., Rubin, B., and Cygler, M. (1993) *J. Biol. Chem. 268,* 12843–12847.
13. Lagone, J.J. (ed.) (1991) *Methods Enzymol. 202,* Academic Press, Inc., New York.
14. Joerger, R.D., and Haas, M.J. (1993) *Lipids 28,* 81–88.
15. Sambrook, J. Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning,* 2nd edn., pp. 1.25–1.30, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
16. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning,* 2nd edn., pp. 1.82–1.84, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
17. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA 74,* 5463–5467.
18. Richardson, L.V., and Richardson, J.P. (1992) *Gene 118,* 103–107.
19. Studier, W.F., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990) in *Methods Enzymol. 185* (Goeddel, D.V., ed.) 60–89, Academic Press, Inc., New York.
20. Deng, T., Noel, J.P., and Tsai, M.-D. (1990) *Gene 93,* 229–234.
21. Viera, J., and Messing, J. (1987) *Methods Enzymol. 153,* 3–11.
22. Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA 82,* 488–492.
23. Sambrook, J. Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning,* 2nd edn., pp. A.1–A.3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
24. D'Alonzo, R.P., Kozarck, W.J., and Wade, R.L. (1982) *J. Am. Oil Chem. Soc. 59,* 292–295.
25. Yamaguchi, S., Mase, T., and Takeuchi, K. (1992) *Biosci. Biotech. Biochem. 56,* 315–319.
26. Clausen, I.G., Svendsen, A., Gormsen, E., Patkar, S., Mondorf, K., Egel-Mitani, M., Barford, M., and Borch, K. (1993) *INFORM 4,* 531 (Abstract RR1).
27. Kouker, G., and Jaeger, K.E. (1987) *Appl. Environ. Microbiol. 53,* 211–213.
28. Svenson, L., Green, R., Joerger, R., Haas, M., Scott, K., Wei, Y., Decewenda, U., Lawson, D.M., and Derewenda, Z.S. (1994) *Proteins: Structure, Function and Genetics 18,* 301–306.

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